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Oxidative stress induced by the photosensitizers neutral red (type I) or rose bengal (type II) in the light causes different molecular responses in *Chlamydomonas reinhardtii*

Beat B. Fischer, Anja Krieger-Liszkay[1], Rik I.L. Eggen*

Department of Environmental Toxicology, Swiss Federal Institute for Environmental Science and Technology (EAWAG), Ueberlandstr. 133, 8600 Dübendorf, Switzerland

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Abstract

The molecular defense mechanisms against photooxidative stress in photosynthetic organisms are essential to protect cells from damaging effects of high light illumination and photoinhibition but also to protect against effects by endogenous and exogenous photosensitizers. Here, we analyzed the genetic response of *Chlamydomonas reinhardtii* to the model type I photosensitizer neutral red (NR) and the type II photosensitizer rose bengal (RB) using DNA-microarrays. Many oxidative and general stress response genes, which were also induced by other oxidative stress conditions, were strongly induced by NR. Only one gene was upregulated by RB, the glutathione (GSH) peroxidase homologous gene *Gpxh*, which was also induced by NR. In addition NR exposure resulted in the reduced expression of most nuclear photosynthetic genes and subunits of the light harvesting complex (LHC) indicating an effect on the photosynthetic activity. This is supported by a stimulation of singlet oxygen generation in NR-treated thylakoids. Thus, in *C. reinhardtii* the *Gpxh* expression is most probably induced by the formation of singlet oxygen in both the NR and RB-treated cells via the activation of a very sensitive and specific sensor, whereas general oxidative stress response mechanisms seem to be involved in the response of most other genes to the type I photooxidative stress.

Keywords: *Chlamydomonas reinhardtii*; Photooxidative stress; Neutral red; Rose bengal; Singlet oxygen; DNA-microarray

1. Introduction

In photosynthetic organisms, the increased formation of reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$) and singlet oxygen ($^1O_2$) is a major source for oxidative stress and cellular damages. Especially in the thylakoid membranes, where the photosynthetic light harvesting complex (LHC) absorbs light energy and drives the electron transport chain in the photosystems I and II (PSI and PSII), increased amounts of ROS are generated by uncontrolled electron transfer reactions [1]. High light intensities enhance the rate of charge recombination and triplet chlorophyll formation in the PSII, resulting in the increased production of $^1O_2$ and photoinhibition [2–5]. Thus, the strict regulation of the excitation of the photosystems or other endogenous photosensitizers, such as porphyrins, and the fast induction of defense mechanisms against oxidative stress is crucial for photosynthetic organisms. Several mechanisms have been described to be involved in the regulation of the responses due to light-induced stresses. Some ROS such as $O_2^{\bullet -}$ and H_2O_2 have been shown to directly act as second messengers to regulate the expression of defense genes, including glutathione (GSH) peroxidases, glutathione-*S*-transferases (GST) and ascorbate peroxidases [1]. For other responses, the redox status of the glutathione or the plastoquinone pool was identified to trigger the response, including the control of gene expression [6–8]. However, unraveling the specific mechanism responsible for the induction of a gene is in

* Corresponding author. Tel.: +41 1 823 5320; fax: +41 1 823 5547.
E-mail address: eggen@eawag.ch (Rik I.L. Eggen).
[1] Institute of Biology II, University of Freiburg, Schänzlestr. 1, 79104 Freiburg, Germany.

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many cases difficult because some of the potential signals, e.g. ROS production and redox status of the plastoquinone pool, are tightly linked during photosynthesis.

By using exogenous photosensitizers, the problem of such linked signals may be partially eliminated. Upon absorption of visible light and entering an excited state, such photosensitizers induce a photooxidative stress independent of the photosynthetic activity. In the presence of oxygen, excited photosensitizers can react in two ways: direct electron transfer to the substrate usually results in a semi-reduced form of the photosensitizer and a semi-oxidized form of the substrate, which becomes fully oxidized upon binding molecular oxygen (type I) [9,10]. Neutral red (NR), a phenazine-based dye widely used for staining cellular particles and as an intracellular pH indicator, has been used as a photosensitizer in phototherapy and was reported to interact with several substrates in a type I reaction [11–13]. The second reaction type involves the transfer of the excitation energy from the photosensitizer to molecular oxygen, resulting in the formation of $^1O_2$ (type II) [10]. Rose bengal (RB) and methylene blue are two typical type II photosensitizers, known to mainly generate $^1O_2$ when excited by light in the visible range [14–16]. Thus, exposure of organisms to RB in the light may specifically induce the genetic response to increased generation of $^1O_2$, whereas NR rather activates the response to free radicals-induced oxidative stress.

Recently, we have shown, that the glutathione peroxidase homologous gene *Gpxh* from *Chlamydomonas reinhardtii* is transcriptionally upregulated by the exogenous photosensitizers RB and methylene blue in the light, most probably through the formation of $^1O_2$ [17,18]. Interestingly, *Gpxh* expression is also strongly induced by the addition of NR under illumination with kinetics that are similar to the RB-induced response, suggesting the presence of a common mechanism for types I and II photosensitizer-induced *Gpxh* expression in *C. reinhardtii*. However, exposure to $O_2^{\bullet -}$, H_2O_2 or organic hydroperoxides only caused a slightly induced *Gpxh* expression, indicating that a specific rather than a general oxidative stress regulates the *Gpxh* transcription. In order to understand the *Gpxh* response in the presence of type I or type II photosensitizers, we wanted to study the NR and RB-induced responses in *C. reinhardtii* and compare these responses with other oxidative stress-induced effects. DNA-microarrays are a good method to compare the genetic response caused by different conditions, enabling to measure the expression levels of large sets of genes in one experiment [19]. In *C. reinhardtii*, the collection of cDNA sequences in an expressed sequence-tag (EST) library has recently led to the development of a first series of DNA-microarrays containing 2876 spots, representing approximately 2700 unique genes of the *C. reinhardtii* nuclear genome [20,21]. We used these microarrays to study the genetic response of *C. reinhardtii* cells exposed to either a type I (NR) or a type II (RB) photosensitizer in the light. Additionally, we compared the response to these photosensitizers with gene expression profiles caused by other oxidative stress conditions. This could give more information about the nature and specificity of the defence mechanisms and the signals, triggering the activation of genes involved in the photooxidative stress response, in particular of the *Gpxh* gene.

2. Materials and methods

2.1. Strains and culture conditions

C. reinhardtii strain $cw_{15}arg_7mt^-$ (CC-1618), generously provided by E. Harris, was inoculated in Tris–acetate–phosphate-medium (TAP) [22] in Erlenmeyer flasks and agitated on a rotatory shaker (150 rpm) under constant illumination (120 μmol m^{-2} s^{-1} PAR) at 25 °C. All media were supplemented with 50 mg/l ampicillin and 50 mg/l arginine.

2.2. Chemicals

NR, RB and menadione (Fluka, Buchs SG, Switzerland) were dissolved in water and stored as 1 mM or 10 mM stock solutions at 4 °C in the dark. H_2O_2 (Merck, Whitehouse Station, USA) and *tert*-butylhydroperoxide (*t*-BOOH) (Fluka, Buchs SG, Switzerland) were stored at 4 °C and were diluted to 1 M stock solutions before use. All chemicals used were of PA quality.

2.3. Growth experiments, stress treatment and RNA isolation

An overnight culture of $cw_{15}arg_7$ mt^- was grown to a cell density of 1×10^7 cells/ml. Then, aliquots of 5 ml of the culture were distributed in six-well culture plates and the appropriate amount of photosensitizer was added. Cell density was analyzed 1, 2, 4 and 6 h after incubation by measuring A_{750} in a one-to-five dilution with water. Average growth rate was calculated for each culture out of the five time point measurements in three independent experiments. In parallel, for each condition one 20 ml culture in a 150 ml Erlenmeyer flask was incubated with the appropriate concentration of photosensitizer for 60 min and total RNA was isolated by the acid guanidine isothiocyanate-phenol-chloroform method [23] using TRIzol Reagent (Invitrogen, Basel, Switzerland) following the suppliers instructions. Sample concentration was adjusted to 3 μg/μl total RNA and RNA quality was checked by agarose gel electrophoreses and ethidium bromide staining.

For all stress treatments with three time point measurements, a culture of strain $cw_{15}arg_7mt^-$ was diluted in 100 ml TAP medium to a cell density of 2×10^6 cells/ml and incubated on a rotatory shaker in the light or in the dark for about 16 h, depending on the experimental purpose. When cultures reached a cell density of about 8×10^6 cells/ml,